Efficient DNA Cleavage Induced by Rh\textsuperscript{III}(TMPyP)\textsuperscript{5+} in the Presence of Ascorbic Acid

Nanda Gunawardhana\textsuperscript{1}, Shingo Homi\textsuperscript{1} & Masaaki Tabata\textsuperscript{1}

\textsuperscript{1} Department of Chemistry, Faculty of Science and Engineering, Saga University, Japan

Correspondence: Masaki Tabata, Department of Chemistry, Faculty of Science and Engineering, Saga University, 1 Honjo-machi, Saga, 840-8502, Japan. Tel: 81-952-288-560. E-mail: tabatam@cc.saga-u.ac.jp

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Abstract

The ability of Rh(III) tetrakis-N-methylpyridyl porphyrin, Rh\textsuperscript{III}(TMPyP)\textsuperscript{5+}, to interact with and cleave DNA was, investigated by UV-visible, luminescence, circular dichroism (CD), electron spin resonance (ESR) and gel electrophoresis methods in the presence or absence of ascorbic acid. UV-absorption data showed that Rh\textsuperscript{III}(TMPyP)\textsuperscript{5+} is capable of interacting with DNA, as indicated by the appearance of a red shift and hypochromicity of the Soret band. The CD data revealed that Rh\textsuperscript{III}(TMPyP)\textsuperscript{5+} was capable of binding to DNA via an external binding mode. The Rh\textsuperscript{III}(TMPyP)\textsuperscript{5+} showed fluorescence and phosphorescence at room temperature. The phosphorescence increased in the presence of DNA and this could be attributed to the shielding of the metal-porphyrin by DNA. Gel electrophoresis studies revealed that Rh\textsuperscript{III}(TMPyP)\textsuperscript{5+} was only able to cleave DNA in the presence of the reducing agent ascorbic acid. ESR data indicated the formation of Rh\textsuperscript{II}(TMPyP)\textsuperscript{5+} by reduction of Rh\textsuperscript{III}(TMPyP)\textsuperscript{5+} with ascorbic acid. The involvement of Rh(III)/Rh(II) species in catalytic DNA cleavage and a possible DNA cleavage mechanism is discussed.

Keywords: Rh\textsuperscript{III}(TMPyP)\textsuperscript{5+}, DNA cleavage, ESR, CD spectra, gel electrophoresis

1. Introduction

Porphyrins and metalloporphyrins have been studied extensively by various research groups because of their uses in photodynamic therapy, cancer detection and virus inhibition (McMillin et al., 2005; Romera et al., 2011; Mező et al., 2011). These complexes readily bind to DNA through three binding modes: electrostatic interactions with the negatively charged sugar-phosphate back bone; intercalation between the base pairs of negatively charged DNA; and interactions within the two grooves of the DNA double helix. ESR, CD, Raman, fluorescence and absorption spectroscopic methods have been widely used to understand these binding modes (Pasternack, 2003). A number of physiochemical methods, e.g., gel electrophoresis, melting temperature measurements and hydrodynamic methods such as sedimentation and viscosity measurements, have also been used to determine the binding modes of porphyrins to DNA (Drexler et al., 1998; Pratviel et al., 1989).

The binding modes of metalloporphyrins to DNA depend on many factors, including experimental conditions such as ionic strength, pH, molar ratio of porphyrin to DNA base pair, the properties of the metal center and the porphyrin core and the ligand structure and/or metal coordination geometry. The pioneering work of Pasternack et al. showed that metalloporphyrins lacking axial ligands will intercalate into DNA and exhibit a negative CD in the Soret band (Pasternack, 2003). In contrast, metalloporphyrins that have axial ligands, such as Zn(II)-, Mn(III)-, Fe(III)-, V(IV)- and Co(III)-TMPyP do not intercalate into DNA (Gibbs et al., 1998; Yellappa et al., 2006; Nyarko et al., 2004). Intercalation is believed to be prevented by axial ligands that sterically impede the insertion of the porphyrin ring between the DNA base pairs. In general, these types of metalloporphyrins bind to DNA via electronic interactions between the negatively charged phosphate groups of DNA and the positively charged porphyrin ring. Previous studies have been conducted with plasmid DNA to understand the relative spectral changes in fluorescence and phosphorescence emission of Pt(II), Pd(II) and Au(III) porphyrins (Nyarko et al., 2004; Tabata et al., 2003; Nyarko et al., 2001; Habib et al., 2004; Tabata et al., 1998).
In an attempt to understand more about the interactions of DNA with metals, especially with noble metals such as Pt(II), Pd(II) and Au(III), we have previously studied the relative luminescence changes these types of metalloporphyrins with DNA (Nyarko et al., 2004). The spectral changes of Rh(III)-porphyrins (d6 systems) are of particular interest due to their apparent similarities to Pd(II), Pt(II) and Au(III)-porphyrins (d8 systems). We have also demonstrated the DNA cleavage mechanisms in the presence of Hg(II), Cd(II) and Pb(II)-porphyrins (Tabata et al., 2003; Nyarko et al., 2001; Habib et al., 2004; Tabata et al., 1998). Based on the knowledge that changing the redox properties of metal center changes the ability to generate reactive oxygen species such as O2 and H2O2, we were able to demonstrate that DNA could be oxidatively damaged by Au(III)-porphyrins. This type of redox activation of metalloporphyrins can be achieved by reducing agents such as ascorbic acid or superoxide anion.

![Scheme 1. Structures of the Rh(III)(TMPyP)5+](image)

Although numerous investigations have been carried out to understand DNA interactions with metalloporphyrins that have axial ligands, only one report has indicated the attraction of DNA to a noble-metalloporphyrin (Nyarko et al., 2004). The present paper reports a DNA cleavage by Rh(III)(TMPyP)5+ that occurs only in the presence of ascorbic acid. A more efficient DNA cleavage was observed for Rh(III)(TMPyP)5+ when compared with other metalloporphyrins. This may be due to involvement of Rh(III)/Rh(II) species for enhanced DNA cleavage. A possible DNA cleavage mechanism is discussed.

2. Experimental

2.1 Materials and Methods

Rhodium (III) chloride trihydrate, sodium chloride, sodium hydroxide, L-ascorbic acid, sodium acetate, ethanol was purchased from Wako Chemicals Co. (Osaka, Japan). 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) was purchased from Dojindo laboratories Ltd., Japan. Moreover, H2(TMPyP)4+ was purchased from Dojindo Laboratories and its Rh(III)-porphyrins were prepared by a standard procedure (Golovina et al., 1998). The pBluescript II plasmid DNA was prepared from a plasmid bearing Escherichia coli strain using a standard procedure and then dissolved in sterilized water. The concentration of base pairs of DNA was determined by absorbance measurements using \( \varepsilon_{260} = 1.32 \times 10^4 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1} \) at the absorption maximum of 260 nm. Doubly deionized water was used throughout the experiments (Milli-QPS TOC, Nippon Millipore Lit. Japan).

Unless otherwise mentioned all experiments were carried out at 25 °C.

2.2 UV-visible Measurements

The UV-visible spectra were acquired on a Shimadzu UV 2100 spectrophotometer. The titrations were made by addition of the DNAstock solution directly to the Rh(III)TMPyP solution at increasing concentrations in base pairs, and the added volume of plasmid DNA was no more than 15 μL to avoid complications due to dilution effects within titration (total volume is 1 mL). The mixture was shaken thoroughly, and then equilibrated for 30 minutes.

2.3 Studies on DNA Damage by Gel Electrophoresis

The standard reaction mixture (in a microtube; 1.5 ml; Eppendorf) contains a buffer agent (HEPES), various concentrations of Rh-porphyrins and plasmid DNA. The mixture was incubated at 37 °C for 60 min in a constant
temperature bath (Yamato, Japan). After incubation, the samples were stained with $1.0 \times 10^{-3}$ ml of a loading buffer (Orange G) and then run in 1% neutral agarose slab horizontal gel containing Tris, acetic acid and EDTA of pH 7.4 in 500 ml doubly deionized water for 30 min. The gel was stained by stirring in a solution of 0.5 $\mu$g l$^{-1}$ of ethidium bromide for 60 min. Gel electrophoresis was performed by means of a Mupid-2 Cosmo Bio Company apparatus (Japan) and DNA bands were photographed with a Polaroid MP-4 land camera using a Polapan black and white coatless film. Moreover, similar gel electrophoresis experiments were carried out with HEPES in the presence of different concentrations of Rh(III)-porphyrins with ascorbic acid as a reducing agent.

2.4 Electron Spin Resonance Measurement

ESR spectra were recorded to detect radicals of Rh species in the presence of ascorbic acid. The spectra were measured at room temperature using a JES-TE 300 (JEOL, Tokyo, Japan) spectrometer. The spectra were recorded with a microwave power of 2 mW and modulation amplitude of 0.63 mT. Moreover, a series of ESR experiments was carried out with different concentrations of Rh$^{III}$TmPyP$^{5+}$ and Rh$^{III}$(Br$_8$TmPyP)$^{5+}$ in the presence of ascorbic acid.

2.5 Circular Dichroism Measurements

Circular dichroism (CD) measurements were conducted with a Jasco J-720 spectropolarimeter (Japan). After each addition of Rh(III)-porphyrin, the spectra were scanned five times at room temperature and then averaged. All experiments were conducted in the presence of 0.10 M sodium chloride. The reagents were added in the order of NaCl, HEPES, DNA, ascorbic acid and Rh(III)-porphyrins. Since the radical formation occurred after the addition of Rh(III)-porphyrin, it was added as a final reagent into the solution.

2.6 Electrochemical Measurements

Electrochemical measurements were carried out in a single-component three-electrode glass cell. A glassy carbon electrode, an Ag/AgCl electrode and a platinum electrode were used as a working electrode, reference electrode and auxiliary electrode, respectively. The glassy carbon (GC) electrode was purchased from Bioanalytical System (area 0.07 cm$^2$) and was pretreated by sequential polishing with 1 and 0.05 $\mu$m of alumina/water slurries on felt pads, followed by rinsing with double distilled water prior to use. Solutions were thoroughly deoxygenated, unless otherwise indicated, by bubbling with nitrogen. During the data acquisition, a nitrogen atmosphere was maintained over the solution in the cell. The supporting electrolyte in this research was 0.1 M tetrabutylammonium hexafluorophosphate solution. CV was performed with an 802A Electrochemical Analyzer (CH Instruments, TX).

3. Results and Discussion

3.1 UV-Visible Spectrum Studies

The binding of porphyrin complexes to DNA causes a red/blue shift, hypso/hyperchromism, or a broadening of the Soret band in UV-Vis spectra. These changes depend on the nature of the DNA, the porphyrin and the binding mode. In general, the spectral changes are large for intercalation and small for groove binding or stacking mode. The absorption spectra of Rh$^{III}$(TmPyP)$^{5+}$ recorded in the presence of increasing amounts of pBluescript plasmid DNA in HEPES buffer, are shown in Figure 1. Upon addition of pBluescript plasmid DNA to Rh$^{III}$(TmPyP)$^{5+}$, the Soret band of Rh$^{III}$(TmPyP)$^{5+}$ was blue shifted from 435 to 431 nm with 12% hypochromicity. A similar observation has been previously made for Co$^{II}$(TmPyP) following addition of plasmid DNA. The intrinsic binding constant, as evaluated from Benesi-Hildebrand plots, was 2.0 x 10$^5$ M$^{-1}$ for Rh$^{III}$(TmPyP)$^{5+}$ (See Figure S1, supporting information). Since the spectral shifts, hypochromicities and binding constant were not in the range of associated with intercalation, this suggested that Rh$^{III}$(TmPyP)$^{5+}$ binds to DNA via an outside binding mode (Pratviel et al., 1989; Zhao et al., 2008; Gibbs et al., 1988). The axial ligand in Rh$^{III}$(Br$_8$TmPyP)$^{5+}$ create steric difficulties for intercalating Rh$^{III}$(TmPyP)$^{5+}$ into DNA under the present experimental conditions.
3.2 Luminescence Studies

The luminescence of metalloporphyrins is related to the presence/absence of d-d transitions lying between the porphyrin $\pi$-$\pi^*$ triplet and the ground state (Vasil'ev et al., 2003). Metalloporphyrin complexes that form with 3d transition metals having low energy exited state of the fields of ligands or charge transfer states do not show luminance. However metalloporphyrins belonging to the platinum group (Pd(II), Pt(II), Rh(III)) emit luminance. The luminance properties of Pd(II), Pt(II) and Rh(III) complexes have been previously studied, along with the generation of singlet oxygen under various conditions. Rh(III)-porphyrins have been demonstrated to show fairly long triplet lifetimes at room temperature; hence, these complexes can be used in photodynamic therapy (Nyarko et al., 2001; Vasil'ev et al., 1999).

Excitation of Rh$^{\text{III}}$(TMPyP)$^{5+}$ in aqueous solution gives rise to very weak fluorescence at 631 nm and moderate phosphorescence at 871 nm. The fluorescence peak is broad where as the phosphorescence peak is quite sharp. The spectrometric titration of a Rh$^{\text{III}}$(TMPyP)$^{5+}$ solution with increasing amounts of pBluescript plasmid DNA provides good information about the interactions of Rh$^{\text{III}}$(TMPyP)$^{5+}$ with DNA. As shown in Figure 2, addition of DNA decreased the fluorescence intensity of Rh$^{\text{III}}$(TMPyP)$^{5+}$. It also caused peak splitting in the spectra, yielding two new peaks at 631 and 649 nm. The relative disappearance of initial fluorescence was 37%. This quenching may be attributed to the self stacking of Rh$^{\text{III}}$(TMPyP)$^{5+}$ along the DNA structure. The same type of behavior has been observed previously for Au$^{\text{III}}$(TMPyP) with DNA. However, the relative intensity of the phosphorescence peak at 871 nm was increased during the titration of Rh$^{\text{III}}$(TMPyP)$^{5+}$ with DNA. As described previously, in the absence of oxygen or other quenchers of phosphorescence, the principle paths of degradation of the energy of electronic excitation are radiative and nonradiative deactivation. However, in an aqueous solution, the phosphorescence of the Rh$^{\text{III}}$(TMPyP)$^{5+}$ is effectively quenched by molecular oxygen. During titration of Rh$^{\text{III}}$(TMPyP)$^{5+}$ with DNA, the marked enhancement of phosphoresce could be attributed to the shielding of Rh$^{\text{III}}$(TMPyP)$^{5+}$ by DNA. This shielding would prevent porphyrin from reacting with the dissolved molecular oxygen in water. Thus, enhancement of phosphorescence is widely accepted as an indication of the interaction between DNA and Rh$^{\text{III}}$(TMPyP)$^{5+}$ via outside binding. Previous studies have been shown that the quantum yield for luminescence varies in the following order: intercalation complex $<$ free porphyrin $<$ external, groove bound complex (Rodriguez et al., 1990). Our results agreed well with reported phosphorescence enhancements and confirmed an out-side binding mode for metalloporphyrins with DNA.
3.3 Circular Dichroism Studies

The CD spectra of porphyrins in the Soret region have been reported as well-defined indicators of the binding modes of porphyrins with DNA. In the absence of DNA, the RhIII(TMPyP)5+ itself does not display CD peaks in the Soret region. However, after interacting with DNA, the achiral porphyrins show different modes in CD spectra. A positive peak in the CD spectrum can be seen when porphyrins bind to the DNA groove; a negative peak can be seen due to intercalation; and a conservative peak can be ascribed to outside binding (Drexler et al., 1998; Pratviel et al., 1989). Figure 3 illustrates the CD spectrum for RhIII(TMPyP)5+ bound to DNA. The ellipticities observed for RhIII(TMPyP)5+ in the presence of DNA are predominantly positive in character and centered at wavelengths ranging from 440 to 460 nm. This positive character became stronger with the increasing amounts of RhIII(TMPyP)5+. In addition, weak negative peak was also observed in the CD spectrum in the range of 420 to 440 nm. These observations suggest that the DNA binding mode for RhIII(TMPyP)5+ is primarily through an external binding, accompanied by partial intercalation or aggregation of RhIII(TMPyP)5+ at a high [DNA]/[RhIII(TMPyP)5+] ratio.

3.4 Electron Spin Resonance Studies

ESR studies can be successfully used to understand the oxidation state, and the site of oxidation or reduction of both the porphyrin macro-cycle and/or the metal center of the metalloporphyrin complexes. ESR studies revealed
that among the various oxidation states of Rh-porphyrins, only Rh(0) (d9), Rh(II) (d7) and Rh(IV) (d5) states are paramagnetic, with S=1/2. The low-spin Rh(III) (d6), does not show an ESR signal. Since hyperfine splitting in the EPR spectra depends on the interaction of the electrons with the 4 nitrogen atoms of the porphyrin macro-cycle and the metal center, it can also be used to analyze the strength of interactions of metalloporphyrins (Kadish et al., 1985; Kadish et al., 1993; Hoshino et al., 1984; Nakamura, et al., 2006). As expected, a fresh sample of Rh(III)(TMPyP)5+ having the Rh(III) oxidation state was ESR silent under the present experimental conditions. However, addition of ascorbic to Rh(III)(TMPyP)5+ showed a new peak in ESR spectra indicating formation of new Rh(III)(TMPyP)5+ species (See Figure S2, supporting information). These results agreed well with the formation of Rh(III)(TMPyP)5+. The Rh(III)(TMPyP)5+ eventually generate a hydroxyl anion radical under the experimental conditions. As shown in Scheme-2, this reduction process enhances DNA scission in aqueous media.

3.5 Gel Electrophoresis Studies

As discussed in materials and methods section, samples containing pBluescript plasmid DNA with Rh(III)(TMPyP)5+ in a HEPES buffer, pH 7.5, were electrophoresed at 37 °C for one hour at different [DNA]/[porphyrin] ratios. The influence of a reducing agent, ascorbic acid, on the reactivity of Rh(III)(TMPyP)5+ was also examined. The cleavage position can be analyzed by agarose gel electrophoresis experiments. After gel electrophoresis experiments, no DNA cleavage was observed with Rh(III)(TMPyP)5+ in the absence of ascorbic acid (data not shown). Acrobic acid does not cleave DNA itself. This non-cleaving behavior of DNA indicates the inability to generate DNA cleavage species by Rh(III)(TMPyP)5+ or ascorbic acid alone under the present experimental conditions. However, as shown in Figure 4, DNA cleavage was induced by Rh(III)(TMPyP)5+ in the presence of ascorbic acid. In Figure 4, lane 1 is a DNA control and lanes 2-6 indicate the decreasing concentrations of Rh(III)(TMPyP)5+ from 10^-4 - 10^-8 mol dm^-3. Lane-2, where Rh(III)(TMPyP)5+ concentration is 10^-4 mol dm^-3, shows a complete conversion of form I of DNA (the supercoiled form) to form II (the relaxed circular form). The intensity of form II band decreased with decreasing the Rh(III)(TMPyP)5+ concentration. As described in Scheme-2, the reduction of Rh(III)(TMPyP)5+ to generate hydroxyl radicals is an important process for DNA scission.

![Form I and Form II](image_url)

Figure 4. Gel-electrophoresis results showing cleavage of DNA by Rh(III)(TMPyP)5+; Lane 1, DNA control; lane 2-6, 1 x 10^-4 to 1 x 10^-8 M Rh(III)(TMPyP)5+ with 0.1 M ascorbic acid. Concentrations of buffering agents and Sodium chloride were maintained at 1 x 10^-2 M and 1 x 10^-2 M, respectively.
To understand more about their reduction potential we then measured CV of RhIII(TMPyP)5+ in DMF solution. The potential corresponding to the reduction process of RhIII(TMPyP)5+ is located at $E_{1/2} = -0.72$ V vs Ag/AgCl. This indicates that RhIII(TMPyP)5+ could be more readily reduced than other reported metal-TMPyP complexes.

![Figure 5. Cyclic voltamgram of RhIII(TMPyP)5+. Scan rate 0.2 V/s. Reference electrode is Ag/AgCl (3.3 M KCl)](image)

The proposed DNA cleavage mechanism for RhIII(TMPyP)5+ with ascorbic acid is shown in Scheme-2. According to the proposed mechanism, the initial step involves reduction of RhIII(TMPyP)5+ to a reduced state, RhII(TMPyP)5+, in the presence of ascorbic acid. The CV and ESR data confirmed formation of this intermediate. The reduced rhodium porphyrin then binds to dioxygen and catalytically reduces it to hydrogen peroxide. The resulting hydrogen peroxide further reacts with the reduced rhodium porphyrin to generate hydroxyl radicals, which in turn cleave the DNA.

\[
\begin{align*}
\text{RhIII(TMPyP)5+} + e^- & \rightarrow \text{RhII(TMPyP)5+} \\
2 \text{RhII(TMPyP)5+} + 2H^+ + O_2 & \rightarrow 2 \text{RhIII(TMPyP)5+} + H_2O_2 \\
H_2O_2 + \text{RhII(TMPyP)5+} & \rightarrow \text{OH}^- + \text{OH}^+ + \text{RhIII(TMPyP)5+} \\
\text{DNA} + \text{OH}^- & \rightarrow \text{DNA fragments}
\end{align*}
\]

Scheme 2. Proposed Mechanism of Reductively Induced RhIII(TMPyP)5+ for DNA Cleavage

4. Conclusion

We were able to demonstrate the interactions of RhIII(TMPyP)5+ with plasmid DNA. UV-vis and CD studies were carried out to determine the nature of binding between RhIII(TMPyP)5+ and plasmid DNA. The relative shift and percent hypochromicity in the Soret band and the binding constant indicate the external binding mode, which was further supported by CD and luminescence studies. The proposed mechanism indicates that catalytic activity is a result of the generation of OH− radicals by reduced form of RhIII(TMPyP)5+, RhII(TMPyP)5+. This mechanism is further supported by gel-electrophoresis results that indicate the occurrence of DNA damage only under reducing conditions. In addition ESR data suggest that the reduced form of RhIII(TMPyP)5+, RhII(TMPyP)5+, is responsible for generating OH− radicals that enhance the DNA cleaving ability.

Abbreviations

HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
ESR: electron spin resonance
CD: circular Dichroism
CV: cyclic voltamogram
SOD: superoxide dismutase
OH−: hydroxyl radical
EDTA: ethylenediaminetetraacetic acid
Tris: tris(hydroxymethyl)aminomethane

Rh\textsuperscript{III}(TMPyP)\textsuperscript{5+}: Rh(III) tetrakis-N-methylpyridyl porphyrin

References


**Supporting Information**

![Figure S-1. Benesi-Hildebrand plot of I/ΔI Versus 1/C constructed for the determination of binding constant of Rh^{III}(TMPyP)^{5+} with DNA](image)

Figure S-1. Benesi-Hildebrand plot of I/ΔI Versus 1/C constructed for the determination of binding constant of Rh^{III}(TMPyP)^{5+} with DNA
Figure S-2. ESR spectrum of Rh$^{III}$ (TMPyP)$^{3+}$ in DMF solution. Concentration of solutions is $1 \times 10^{-5}$ M. Concentration of ascorbic acid is 1.0 M